Immunity, Vol. 12, 263–272, March, 2000, Copyright ©2000 by Cell Press

# A Proposed Mechanism for the Induction of Cytotoxic T Lymphocyte Production by Heat Shock Fusion Proteins

**Bryan K. Cho,*‖ Deborah Palliser,*‖ Eduardo Guillen,‡ Jan Wisniewski,‡ Richard A. Young,*† Jianzhu Chen,* and Herman N. Eisen*§**

*Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
†The Whitehead Institute
Cambridge, Massachusetts 02139
‡Stressgen Biotechnologies Inc.
Victoria VB2 4B9
Canada

## Summary

**A 65 kDa mycobacterial heat shock protein (hsp65), fused to a polypeptide that contains an octapeptide (SIYRYYGL) agonist for a particular T cell receptor (2C TCR), stimulated C57BL/6 mice as well as CD4-deficient mice to produce $CD8^+$ cytolytic T lymphocytes (CTL) to the fusion partner's octapeptide. This and other hsp65 fusion proteins but not native hsp65 itself stimulated dendritic cells in vitro and in vivo to upregulate the levels of MHC (class I and II) and costimulatory (B7.2) molecules. The results suggest a mechanism for the general finding that hsp fusion proteins, having fusion partners of widely differing lengths and sequences, elicit CD8 CTL to peptides from the fusion partners without requiring exogenous adjuvants or the participation of $CD4^+$ T cells.**

## Introduction

When injected with diverse adjuvants, protein antigens usually stimulate the production of high-affinity IgG antibodies, indicating that they activate CD4 T helper cells as well as B cells. These procedures generally fail, however, to elicit effective CD8 T cell responses. The reason, according to current views, is that the short peptides that are needed, in association with MHC class I molecules, to stimulate CD8 T cells arise from proteolytic cleavage of cytosolic proteins. Since injected protein antigens are generally unable to cross cellular lipid membranes, they fail to gain entry to the proper cytosolic "MHC class I processing pathway" and are thus unable to stimulate the production of CD8 T cells. Although there is evidence of alternative cellular pathways for processing some exogenous proteins to form peptide MHC class I complexes (Gromme et al., 1999; Sigal et al., 1999), it remains generally true that protein antigens normally fail to stimulate significant CD8 cytolytic T lymphocytes (CTL) responses (Rock, 1996).

Mycobacterial heat shock proteins (hsp) fused with large protein fragments termed fusion partners may be among the few exceptions to this rule. When injected into mice in saline solution (PBS) without added adjuvants, several of these fusion proteins were previously shown to stimulate the production of CD8 CTL that recognize short peptide epitopes (8–10 amino acids in length) that arose from the fusion partners. The fusion partners varied from ~80 to 110 amino acids in length and were derived from ovalbumin (Suzue et al., 1997), influenza virus nucleoprotein (Anthony et al., 1999), and an entire protein subunit of a human papilloma virus (N. R. Chu, personal communication). To explore the mechanisms that permit these hsp to be effective with such diverse fusion partners and that enable the hsp fusion proteins to serve as effective immunogens for CD8 T cells without requiring adjuvants, we studied the immunogenic activities of fusion proteins prepared from the 65 kDa hsp from *Mycobacterium bovis*, BCG strain (here called hsp65).

The principal fusion partner used in this study is a polypeptide that contains an octapeptide sequence, SIYRYYGL (hereafter called SYRGL; Udaka et al., 1996), which together with $K^b$ serves as a potent stimulator of CD8 T cells having the TCR of a CTL clone called 2C (Kranz et al., 1984). This peptide was identified in a synthetic peptide library and, so far as is known, does not occur in nature. We have relied here on the use of various T cells that express 2C TCR, particularly naive 2C T cells (Cho et al., 1999), as specific probes to obtain evidence that (1) dendritic cells are more effective than macrophages in presenting the processed hsp fusion protein to naive CD8 T cells, (2) dendritic cells are stimulated directly by each of several hsp65 fusion proteins tested but not by "native" hsp65 itself to increase surface expression of MHC class I and II and costimulatory (B7.2) molecules, and (3) CD4 T cells are not required for the fusion protein's ability to elicit production of CD8 CTL in vivo. Taken together, the results begin to suggest an explanation for the general finding that diverse soluble heat shock fusion proteins, regardless of the length or sequence of the fusion partners, stimulate CD8 T cell responses to peptides derived from the fusion partners without requiring exogenous adjuvants. The findings are of particular interest in view of the need to develop protective vaccines against intracellular pathogens for which current immunization strategies are inadequate (e.g., against HIV-1, human papilloma virus, various herpes viruses, and malaria).

## Results

### Design and Characterization of Heat Shock Fusion Protein hsp65-P1

As shown in Figures 1A and 1B, the principal fusion protein used in this report contains the polypeptide P1 fused to the C terminus of hsp65. P1 includes the octapeptide SYRGL, which behaves in association with $K^b$ as a strong agonist for the TCR on 2C T cells (Sykulev et al., 1998). The sequences that flank the octapeptide in P1 were chosen because they correspond to those known to be effectively cleaved intracellularly in two un-

§To whom correspondence should be addressed (e-mail: hneisen@mit.edu).

‖These authors contributed equally to this work.

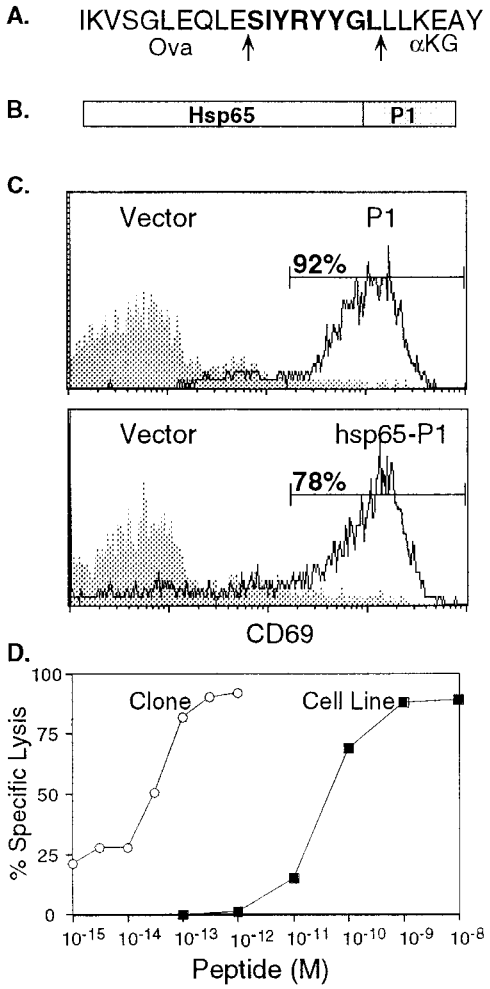


Figure 1. Design and Characterization of Heat Shock Fusion Protein hsp65-P1

(A) P1 peptide. When liberated from P1, SIYRYYGL (boldface) binds to $K^b$ to form the peptide–MHC complex recognized by the 2C TCR. In P1, SIYRYYGL is flanked 5′ and 3′ by sequences that lie immediately upstream and downstream, respectively, of peptide bonds that are cleaved (see arrows) in murine cells to liberate naturally occurring peptides (SIINFEKL from ovalbumin [Ova] and LSPFPFDL from α-ketoglutaraldehyde dehydrogenase [aKG]) (Falk et al., 1992; Udaka et al., 1992).

(B) Diagram of the hsp65-P1 fusion protein, showing P1 at the C terminus of hsp65.

(C) Evidence that P1 and hsp65-P1 are processed intracellularly to yield the SYRGL octapeptide. Forty-eight hours after transfection with mammalian expression vectors (VR1055 and pCINeo), containing sequences that encode P1 and hsp65-P1, respectively, EL4 cells were incubated for 18 hr with an equal number of naive 2C T cells. Histograms show the percentage of live, $2C^+CD8^+$ cells that were stimulated to upregulate the activation marker CD69. The responses of these naive T cells to control EL4 cells, transfected with the empty (Vector) plasmids, are shown as shaded histograms.

(D) Normal C57BL/6 mice have T cells that can recognize the SYRGL–$K^b$ complex. A $CD8^+$ T cell line, derived from C57Bl/6 mice immunized with the SYRGL peptide in adjuvant, specifically lysed T2-$K^b$ target cells in a SYRGL-dependent manner. A highly cytolytic long-term cultured 2C CTL clone (L3.100) is shown for comparison.

related proteins: ovalbumin (Falk et al., 1992) and α-ketoglutaraldehyde dehydrogenase (Udaka et al., 1992; Figure 1A, see arrows). To determine if the P1 polypeptide, alone or linked as a fusion partner to hsp65, could be cleaved intracellularly to liberate the SYRGL octapeptide, we transfected plasmids containing sequences for P1 or hsp65-P1 into EL4 cells (H-$2^b$). Because relatively few of the transiently transfected cells were expected to express P1 or hsp65-P1, the transfected cell population was not used in cytolytic assays as targets for 2C CTL. Instead, we examined their ability to stimulate naive 2C T cells. As shown in Figure 1C, 80%–90% of these naive T cells were stimulated to express the acute activation marker CD69 in response to EL4 cells transfected with either the P1 or hsp65-P1 plasmids, while virtually none of the naive T cells were activated by cells transfected with the empty plasmids (Figure 1C, "Vector," shaded histograms). These results indicate that in these transfected cells, P1 and hsp65-P1 can be cleaved to release the octapeptide, which is then presented by $K^b$.

### C57BL/6 Mice Produce SYRGL-Specific $CD8^+$ Cytolytic T Cells in Response to hsp65-P1

Before immunizing mice with hsp65-P1, we first had to be sure that CD8 T cells that can recognize the SYRGL octapeptide are present in normal C57BL/6 (H-$2^b$) mice. The mice were therefore injected with SYRGL peptide in adjuvant (TiterMaxGold), and their spleen cells were maintained in culture for several weeks (see Experimental Procedures) and subsequently tested in a standard cytotoxicity assay. As shown in Figure 1D, the cell line's lysis of $K^{b+}$ target cells (T2-$K^b$) was SYRGL dependent, indicating the presence in these mice of T cells that can respond to SYRGL–$K^b$ complexes.

To determine if the hsp65-P1 fusion protein could stimulate ("prime") anti-SYRGL CTL in vivo, normal C57BL/6 mice were injected subcutaneously with the fusion protein in saline without added adjuvants. Each mouse received two injections, 1 week apart. Seven days after the second injection, cells from regional lymph nodes and spleen were restimulated in culture with SYRGL (1 μM) in the absence of exogenous cytokines and tested after 6 days for CTL activity in a 4 hr cytolytic assay, using $^{51}Cr$-labeled $K^{b+}$ target cells (T2-$K^b$; see Experimental Procedures). Of 40 injected mice, 35 produced CTL whose lysis of the $K^{b+}$ target cells was SYRGL dependent (see Figure 2A for a representative response). C57BL/6 mice treated in exactly the same way with equimolar amounts of various controls (hsp65, P1, a mixture of hsp65 and P1, or SYRGL alone) all failed to yield SYRGL-specific CTL (Figure 2A). As little as 1 μg (0.015 nmol) of hsp65-P1 could elicit an anti-SYRGL CTL response. A control fusion protein made by fusing the P1 sequence to the C terminus of another bacterial protein, chosen simply for ease of purification (the *E. coli* maltose binding protein [Mal-P1]), was ~10–100 times less effective in these assays (Figure 2B) and without any detectable effect in others (Figures 3–6). Removal of CD8 T cells by magnetic sorting showed that the cytolytic response to hsp65-P1 was due to CD8 T cells (Figure 2C). These results demonstrate that hsp65-P1, without added adjuvants, can elicit a CD8 T cell response to the fusion partner.

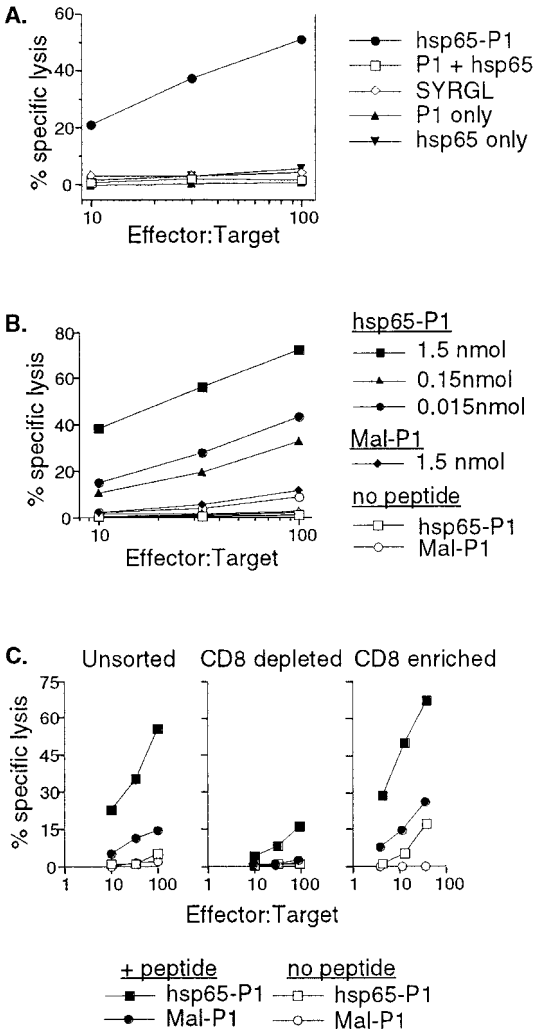


Figure 2. Response of C57BL/6 Mice to hsp65-P1

(A) CD8 CTL that recognize the SYRGL–$K^b$ complex are produced in C57BL/6 mice injected with hsp65-P1 in PBS but not in those injected similarly with equimolar amounts of various controls (a mixture of P1 and hsp65, the SYRGL octapeptide, the P1 polypeptide itself, hsp65 itself).

(B) SYRGL-specific CTL in mice injected as in (A) with various amounts of hsp65-P1, 0.015–1.5 nmol (1–100 μg), or a control fusion protein in which P1 is linked to the C terminus of a maltose-binding protein (Mal-P1, 1.5 nmol, 80 μg).

(C) Depletion of $CD8^+$ T cells eliminates the SYRGL-specific CTL produced by mice injected with hsp65-P1. Lymph node and spleen cells from C57BL/6 mice immunized with 1.5 nmol of hsp65-P1 or Mal-P1 were cultured for 6 days and then depleted of CD8 T cells by magnetic sorting. The untreated, CD8-depleted, and CD8-enriched populations (30%, 1%, and 90% $CD8^+$ T cells, respectively) were analyzed in a 4 hr cytolytic assay.

In (A), (B), and (C) target cells are T2-$K^b$ plus 1 μM SYRGL. In (B) and (C) lysis of T2-$K^b$ target cells in the absence of added SYRGL peptide is indicated by unfilled symbols.

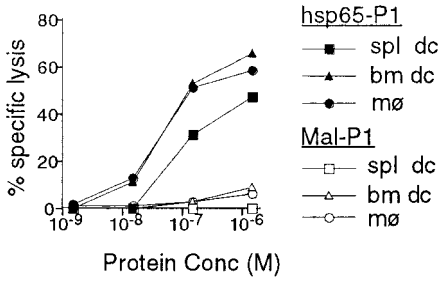


Figure 3. Macrophages and Dendritic Cells Incubated Briefly with hsp65-P1 Become Target Cells for a SYRGL-Specific CTL Clone

$^{51}Cr$-labeled splenic dendritic cells ("spl dc"), bone marrow–derived dendritic cells ("bm dc"), or purified macrophages ("mϕ") isolated from peritoneal lavage, all from B6 (H-$2^b$) mice, were incubated for 4 hr with a 2C CTL clone (L3.100; see Figure 1D) and various concentrations of hsp65-P1 or Mal-P1. CTL:target cell ratio (E:T) of 5:1. Unfilled symbols show lysis when the control fusion protein (Mal-P1) was used in place of hsp65-P1.

## Dendritic Cells and Macrophages Differ in Ability to Serve as Antigen-Presenting Cells for hsp65-P1

To identify antigen-presenting cells (APC) that mediate in vivo CD8 T cell responses, purified preparations of APC from C57BL/6 mice (dendritic cells from spleen or bone marrow and macrophages from peritoneal lavage) were tested for their ability to present processed hsp65-P1 and serve as target cells in cytolytic assays, using a well-established SYRGL-$K^b$-specific CTL clone (L3.100) as an effector. When dendritic cells and macrophages were $^{51}Cr$ labeled and incubated with hsp65-P1 for 4 hr, they were lysed effectively and to about the same extent (Figure 3). No significant lysis was observed, however, when the control fusion protein Mal-P1 was used in place of hsp65-P1, suggesting that processing of hsp65-P1 by these APC was not due to indiscriminate extracellular proteolysis.

Cytolytic reactions with potent CTL clones such as L3.100 can be exquisitely sensitive, detecting very few and perhaps as few as one cognate peptide–MHC complex per target cell (Sykulev et al., 1996). Therefore, we used a more discriminating assay in which dendritic cells and macrophages that had been incubated with hsp65-P1 were compared for their ability to stimulate naive 2C T cells. As shown in Figure 4, when the dendritic cells were incubated with hsp65-P1 overnight and then with naive 2C T cells, the naive T cells were stimulated to (1) express CD69, (2) proliferate, and (3) secrete IL-2 and IFNγ. In contrast, the macrophage preparations stimulated none of these responses. (It may be that activated macrophages would have behaved differently, but we deliberately focused on nonactivated macrophages and dendritic cells to simulate conditions in the unimmunized animal.) The responses elicited by dendritic cells could be inhibited by the clonotypic, anti-2C TCR antibody (1B2; Figure 4C), indicating that they were mediated by ligation of the 2C TCR. The requirement for the hsp65 moiety in the hsp65-P1 fusion protein is emphasized by the result that the naive 2C T cells were stimulated to express CD69 by dendritic cells that had been incubated with hsp65-P1 but not by those that had been incubated with the control fusion protein Mal-P1 (Figure 4A).

Incubation of dendritic cells with various controls (P1 alone, hsp65 alone, or a mixture of hsp65 plus P1) in place of hsp65-P1 did not stimulate 2C T cells to secrete IFNγ. However, of all the controls, the P1 peptide was exceptional in that it exhibited some activity: with both

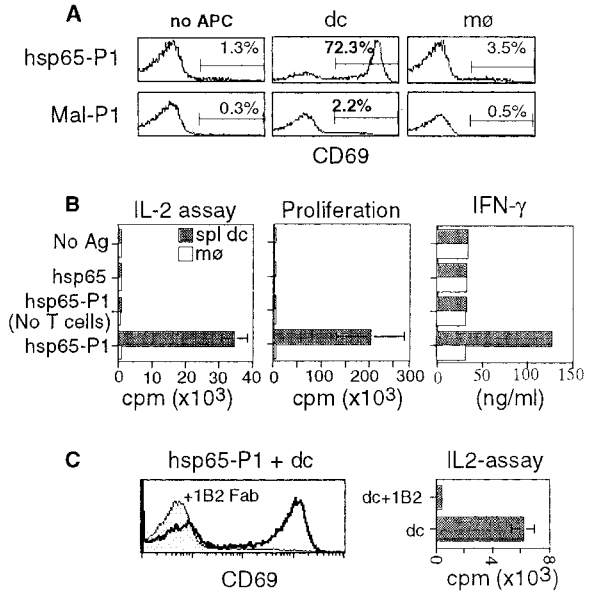


Figure 4. Dendritic Cells but Not Macrophages Generate Sustained Responses from Naive 2C T Cells to hsp65-P1

Splenic dendritic cells and peritoneal lavage macrophages were purified by magnetic sorting and incubated for 18–24 hr with equimolar concentrations of hsp65-P1 or Mal-P1 (in [A]) and hsp65-P1 or unmodified hsp65 (in [B]) before adding naive 2C T cells.

(A) Expression of the activation marker CD69. Hsp65-P1 or Mal-P1 were added to purified splenic dendritic cells, macrophages, or media alone (no APC) at 15 nM (~1 μg/ml). After 24 hr, purified naive 2C T cells were added (T cell:APC ratio of 1:1), and 18 hr later cells were analyzed for CD69, gating on propidium iodide–negative 2C+CD8$^+$ cells. The percentage of 2C T cells with increased expression of CD69$^+$ is indicated.

(B) Dendritic cells or macrophages were incubated with hsp65-P1 or hsp65 before adding the naive 2C T cells as in (A), and incubation was continued for an additional 18 hr (IL-2 assay), 60 hr (proliferation assay), or 48 hr (IFNγ assay). "No Ag" means the dendritic cells and 2C T cells were present but hsp65-P1 and hsp65 were absent; "No T cells" means hsp65-P1 and APC were present but the 2C T cells were omitted.

(C) Inhibition of responses by a clonotypic monoclonal antibody to the 2C TCR (IB2). Bone marrow–derived dendritic cells were incubated with 10 μg/ml hsp65-P1 overnight. Equal numbers of naive 2C T cells were then added in the presence or absence of 1B2 Fab fragments (25 μg/ml). After an additional 18 hr, cells and supernatants were analyzed, respectively, for CD69 expression (left panel) and IL-2 production ([$^3$H]thymidine incorporation by IL-2-responsive HT2 cells, right panel).

dendritic cells and macrophages it stimulated CD69 expression, and with dendritic cells but not with macrophages it induced proliferation and IL-2 secretion by the naive 2C T cells (data not shown). It is likely that the P1 peptide itself is subject to proteolysis by these APC, particularly by dendritic cells, but whether extracellularly or in some intracellular compartment is not clear. Whatever the explanation, it should be noted that the P1 polypeptide did not stimulate CD8 CTL production in vivo under conditions in which the hsp65-P1 fusion protein was consistently effective (Figure 2A). In addition, as is shown later, P1 failed to activate dendritic cells (see Figure 6 below).

To examine the difference between dendritic cells and macrophages more closely, we incubated these cells with various concentrations of the fusion proteins and then evaluated their ability to stimulate naive 2C T cells. As shown in Figure 5A, the naive cells proliferated and produced substantial amounts of IL-2 in response to dendritic cells that had been incubated with concentrations of hsp65-P1 in the 0.1–.01 μM range. In contrast, the responses by naive cells were negligible when macrophages were used in place of dendritic cells or when the Mal-P1 control fusion protein was used at concentrations of up to 1 μM (Figure 5A). Together, these data suggest that dendritic cells are more effective than macrophages in processing and presenting the octapeptide from hsp65-P1.

### Heat Shock Fusion Proteins Stimulate Dendritic Cells Directly

The distinctive ability of hsp65-P1 to stimulate naive 2C (anti-SYRGL) T cells in vitro only in the presence of dendritic cells led us to examine the effect of hsp65-P1 on dendritic cells directly. As shown in Figure 6A, when immature bone marrow–derived dendritic cells (day 6 in culture) were incubated overnight with various concentrations of hsp65-P1, the dendritic cell surface level of an MHC class I molecule (K$^b$) was increased. The extent of the increase depended on the hsp65-P1 concentration, and no increase was seen when hsp65-P1 was replaced by a series of control proteins and peptides (hsp65 alone, P1 alone, SYRGL, Mal-P1, or a monoclonal IgG antibody [anti-2,4,6, trinitrophenyl]).

Other hsp65 fusion proteins, having various fusion partners (influenza virus nucleoprotein or the E7 subunit of human papilloma virus), also elicited increased expression of K$^b$ on the dendritic cells (Figure 6A). It is important to note, however, that unmodified hsp65 ("hsp65 only" in Figures 6A and 6B) consistently failed to stimulate dendritic cell upregulation of K$^b$.

All of the fusion proteins as well as unmodified hsp65 were produced as recombinant proteins in *E. coli* and contained trace levels of endotoxin (lipopolysaccharide [LPS]). An endotoxin standard by itself evoked a weak response at the highest concentration tested (5 endotoxin units [EU]/ml, Figure 6B). Because of molecular weight heterogeneity of LPS, conversion of endotoxin units into LPS weight and mole units is highly approximate. But, if one EU corresponds to about 5 ng LPS and the "average" molecular weight of LPS is taken to be ~10,000, LPS would appear to be somewhat more effective than hsp65 fusion proteins in activating dendritic cells. Nevertheless, the effects of the fusion proteins seemed clearly not to be due to endotoxin contaminants, because when hsp65-P1, hsp65, or Mal-P1 were each added in amounts that resulted in addition of equivalent EU units to the dendritic cells, increased expression of K$^b$ was elicited only by hsp65-P1 (Figure 6B). Moreover, when the data from Figure 6A were plotted against the EU concentrations attributable to the controls and fusion proteins, it was evident that each of the four hsp65 fusion protein preparations but none of the controls stimulated increased expression of MHC class I protein. Finally, all the hsp65 fusion proteins elicited increases in MHC class I expression on dendritic cells from C3H/HeJ mice, a strain known to be unresponsive to LPS (due to a mutation in the Toll4 receptor) (Poltorak et al., 1998). Taken together, the findings demonstrate that hsp65 fusion proteins can activate dendritic cells.

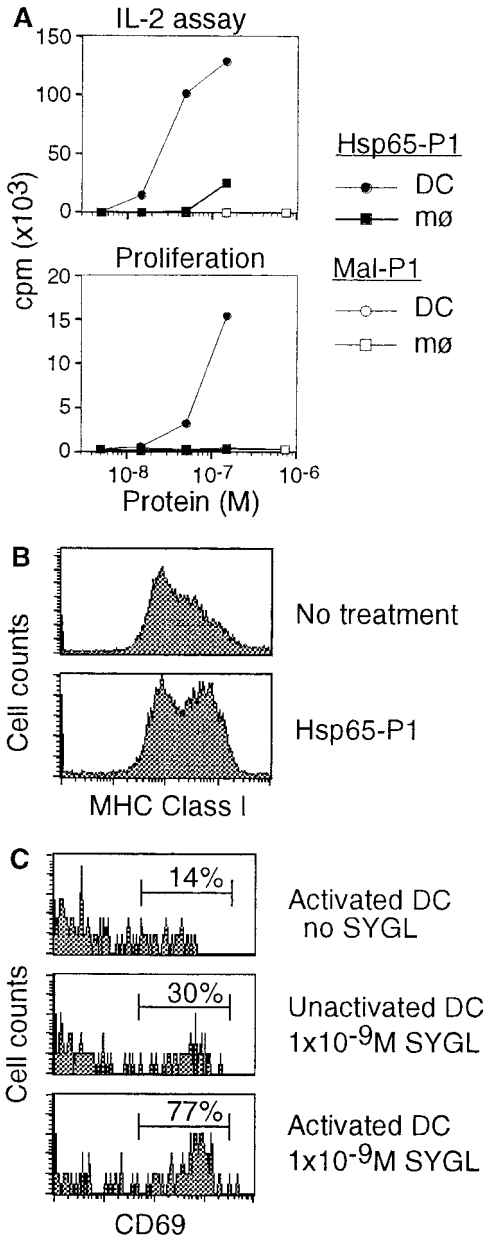


Figure 5. Comparison of Dendritic Cells' and Macrophages' Ability to Stimulate T Cell Responses at Limiting Antigen Dose In Vitro, and Behavior of hsp65 Fusion Protein–Activated Dendritic Cells in Vivo

(A) Fresh splenic dendritic cells or macrophages were incubated with various concentrations of hsp65-P1 or Mal-P1 fusion proteins for about 18 hr before adding purified naive 2C T cells (see Figure 4). Supernatants were sampled 18 hr later to determine IL-2 levels (upper panel). [$^3$H]thymidine was added at 48 hr, and cells were harvested after an additional 18 hr to assess T cell proliferation (lower panel).

(B) Activation of dendritic cells in vivo. Myeloid dendritic cells from lymph nodes draining a subcutaneous site where hsp65-P1 was injected 24 hr previously show increased expression of MHC-I ($K^b$) (lower panel) compared to myeloid dendritic cells from lymph nodes draining an uninjected site (no treatment, upper panel)

(C) Dendritic cells activated with a noncognate hsp fusion protein (hsp65-NP) and pulsed with $10^{-9}$ M SYRGL peptide are more effective than nonactivated, similarly pulsed dendritic cells in stimulating naive T cells in vivo. Dendritic cells ($8 \times 10^5$) were injected into a hind footpad of normal B6 mice that had been injected intravenously with $2 \times 10^6$ naive 2C TCR+ cells (from 2C TCR transgenic RAG-deficient mice). Twenty-four hours after the footpad injection, 2C $CD8^+$ T cells in the draining popliteal lymph node were examined for CD69 expression. Frequency of $CD69^+$ 2C $CD8^+$ T cells in a lymph node draining the site where activated (control) dendritic cells (not pulsed with peptide) were injected (upper panel) or where SYRGL peptide-pulsed ($1 \times 10^{-9}$ M) unactivated dendritic cells or activated dendritic cells were injected (middle panel and lower panel, respectively). Percentages of $CD69^+$ 2C cells are shown. Geometric mean fluorescence values for MHC-I ($K^b$) on dendritic cells that had been incubated prior to footpad injection with or without hsp65-NP were 379 and 97, respectively (data not shown).

Besides stimulating the dendritic cells (bone marrow derived and maintained in culture with GM-CSF for 6 days) to express increased levels of MHC class I, the hsp fusion proteins stimulated increased expression of MHC class II and B7.2 (CD86) (Table 1); the level of CD40 was, however, only marginally affected. Native hsp65 did not affect expression of MHC class II or B7.2, just as it failed to affect levels of MHC class I.

### Activated Dendritic Cells In Vivo

That the dendritic cell changes could also be elicited in vivo was indicated by the finding that 24 hr after injecting hsp65-P1 (in saline) subcutaneously into mice, myeloid dendritic cells (but not lymphoid dendritic cells) from lymph nodes draining the site of injection showed increased expression of $K^b$ (Figure 5B).

To determine if activated dendritic cells were especially effective in vivo, normal B6 mice were adoptively transferred with $2 \times 10^6$ naive 2C cells (from 2C TCR transgenic mice; Cho et al., 1999), and the next day the recipients were injected in a hind footpad with $8 \times 10^5$ dendritic cells. The dendritic cells had been incubated overnight with or without hsp65-NP (to generate activated or nonactivated dendritic cells, respectively) and then incubated for 2 hr with SYRGL peptide at various concentrations (0, $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$ M) and washed just before the cells were injected. Twenty-four hours later, 2C $CD8^+$ T cells from the draining popliteal lymph nodes were examined for CD69 expression as evidence of having been antigenically stimulated. As shown in Figure 5C, when the peptide concentration was $10^{-9}$ M, the activated dendritic cells were considerably more effective than the nonactivated dendritic cells in stimulating the naive 2C T cells to express CD69. When pulsed with the peptide at 10–1000 times higher concentrations, activated and nonactivated dendritic cells were about equally effective, stimulating CD69 responses of the 2C $CD8^+$ cells at about the level seen in Figure 5C, bottom panel.

### Stimulation of CD8 CTL Production In Vivo by the hsp65-P1 Fusion Protein Does Not Require the Participation of CD4 T Cells

The ability of the hsp fusion proteins to directly stimulate dendritic cells suggested that CD4 T cells might not be necessary for the CD8 T cell response elicited in vivo by the fusion proteins. To test this possibility, we immunized CD4 knockout mice ($CD4^{-/-}$) using the same regimen as before (Figure 2) and assessed their ability to produce SYRGL-specific CTL. As seen in a representative response in Figure 6C, the $CD4^{-/-}$ mice produced CTL in response to hsp65-P1 but not in response to the

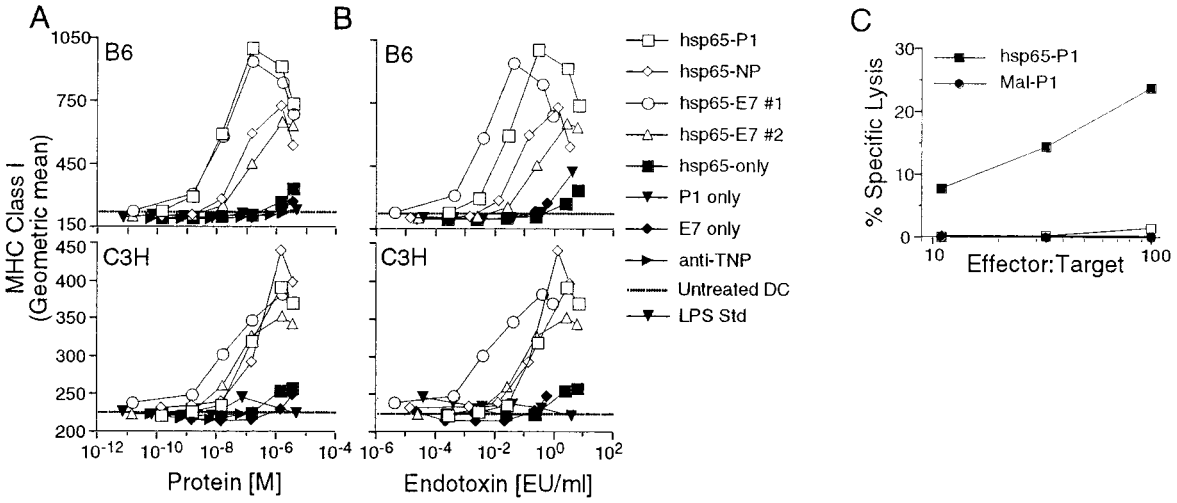


Figure 6. Hsp65 Fusion Proteins Can Directly Stimulate Dendritic Cells and hsp65-P1 Elicits CD4 T Cell–Independent Production of CTL

Purified bone marrow–derived dendritic cells were incubated for 24 hr with various concentrations of hsp65-P1 or other hsp65 fusion proteins, having as fusion partners influenza virus nucleoprotein (hsp65-NP) or human papilloma virus, type 16, E7 subunit (hsp65-E7 preparations 1 and 2) or with controls (hsp65 alone, P1 alone, E7 alone, an anti-TNP IgG antibody). MHC class I protein levels on the dendritic cells were then determined by flow cytometry by gating on propidium iodide–negative $CD11c^+$ cells and using the Y3 antibody which recognizes both H-$2^b$ ($K^b$) and H-$2^k$ MHC class I. MHC class I levels are shown as geometric mean fluorescence; the levels on untreated dendritic cells are represented by a dashed horizontal line.

(A) Dendritic cell MHC class I expression plotted as a function of protein concentration of the added hsp fusion proteins and control proteins. Upper panel shows dendritic cells from C57BL/6 mice. Lower panel shows dendritic cells from C3H/HeJ mice.

(B) The dendritic cell MHC class I expression values from (A) are plotted as a function of endotoxin concentration (calculated from the endotoxin levels present in the added hsp fusion proteins and other proteins). Upper panel shows dendritic cells from C57BL/6 mice. Lower panel shows dendritic cells from C3H/HeJ mice.

(C) Hsp65-P1 stimulates production of CTL (anti-SYRGL) in CD4-deficient ($CD4^{-/-}$) mice. As in Figure 2, the mice were injected subcutaneously twice, one week apart, with 100 μg of hsp65-P1 or Mal-P1 in PBS. One week following the second injection, cells from spleen and draining lymph nodes were pooled and restimulated with 1 μM SYRGL peptide without addition of exogenous cytokines. Six days later the cells were used as effectors in a standard 4 hr cytolytic assay at various E:T ratios using $^{51}Cr$-labeled T2-$K^b$ cells as targets in the presence of 1 μM SYRGL. Lysis of T2-$K^b$ cells in absence of SYRGL is shown by unfilled symbols.

control Mal-P1. While the cytolytic activity elicited in the $CD4^{-/-}$ mice (n = 6) was unambiguous, it appeared to be somewhat less than was generally elicited in normal C57BL/6 mice. In other experiments, C57BL/6 mice that had been extensively depleted of CD4 cells by repeated injections of an anti-CD4 mAb (GK1.5) also responded to the standard immunization protocol with hsp65-P1 about as well as untreated normal mice (data not shown). All of these results show that in stimulating CD8 CTL production in mice, hsp65-P1 does not require the participation of CD4 T cells. The findings do not exclude the possibility, however, that CD4 T cells can enhance the response in normal individuals. Similar evidence that CD4 T cells are not essential for the murine CD8 CTL response to a different hsp fusion protein (*M. tuberculosis* hsp70 fused at its N terminus to a fragment of ovalbumin) has been obtained in an independent study (Huang et al., 2000).

## Discussion

In this report we show that mycobacterial hsp65 fused to the P1 polypeptide activates dendritic cells and stimulates, in the absence of $CD4^+$ T cells, the production of $CD8^+$ CTL that recognize a short peptide derived from P1. The findings extend the number and diversity of hsp fusion proteins that can elicit CD8 T cell responses and suggest a potential mechanism by which the fusion proteins exert their effects in the absence of added adjuvants, a prominent feature of the in vivo responses to all hsp fusion proteins. Generally, where CD4 T cells and adjuvants are required for CD8 T cell responses, it is likely that they function by activating dendritic cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). The capacity of heat shock fusion proteins to directly activate dendritic cells may account for their ability to bypass the requirements for CD4 T cells and added adjuvants.

### Hsp Fusion Proteins Activate Dendritic Cells Directly

Using CTL to detect polypeptide processing by APC, previous studies pointed to macrophages or equally to macrophages and dendritic cells as being responsible for processing protein immunogens that elicit CD8 T cell responses (Kovacsovics-Bankowski and Rock, 1995; Suto and Srivastava, 1995). We also found that when macrophages and dendritic cells were incubated with hsp65-P1, they became equally susceptible to lysis by peptide-specific CTL in a standard 4 hr cytolytic assay, indicating that both types of APC could generate small peptides from the hsp fusion protein and load them on to MHC class I molecules. However, when these cells were evaluated for their ability to stimulate naive CD8

Table 1. Heat Shock Fusion Proteins Stimulate Increased Expression of MHC and Costimulatory Molecules on Dendritic Cells

| | Nothing | Unmodified hsp65 | P1 Peptide | hsp65-NP | hsp65-P1 |
|---|---|---|---|---|---|
| MHC Class I | 100 | 105 | 96 | 257 | 151 |
| MHC Class II | 63 | 67 | 55 | 279 | 90 |
| B7.2 | 60 | 54 | 47 | 129 | 81 |
| CD40 | 45 | 45 | 47 | 76 | 51 |

Dendritic cells derived from bone marrow of C3H/HeJ mice were incubated for 18 hr with $1.5 \times 10^{-6}$ M of various heat shock fusion proteins or the control P1 peptide prior to cell surface staining. Numbers are mean fluorescence values.

T cells to proliferate and produce IL-2 and IFNγ, the dendritic cells but not the macrophages proved to be effective. A step toward understanding this difference comes from the present finding that hsp65-P1 as well as each of the other hsp65 fusion proteins tested is capable of directly stimulating dendritic cells to increase their surface expression of MHC class I and II and costimulatory (B7.2) molecules.

Dendritic cells infected with mycobacteria (including BCG), streptococci, or Leishmania have been shown to upregulate MHC and costimulatory molecules B7.1 and B7.2 and, in addition, to secrete IL-12 (Henderson et al., 1997; Rescigno et al., 1998 Demangel et al., 1999; Konecny et al., 1999). It may be that microbial cell hsp molecules are responsible for these effects. If so, our findings (of a difference between hsp65 fusion proteins and unmodified hsp65) suggest that upregulation of these activation molecules may be due to the hsp in a modified form, resembling perhaps the hsp65 fusion proteins studied here rather than native hsp molecules.

Dendritic cells infected with certain viruses, e.g., influenza virus (Ridge et al., 1998), likewise become activated. However, the hsp fusion proteins appear so far to be the only soluble immunogenic proteins that directly activate dendritic cells in vitro and in vivo to upregulate expression of MHC and costimulatory molecules. Our experimental system should be useful for investigating the pathways by which hsp fusion proteins are processed and presented by dendritic cells and the mechanisms by which MHC and costimulatory molecules are upregulated.

Diverse hsp bind peptides noncovalently, and studies of several hsp (gp96, hsp70, calreticulin) have shown that they can stimulate CD8 CTL production to bound peptides (Srivastava, 1993; Nair et al., 1999). Since these hsp–peptide complexes exert their immunogenic effects without requiring exogenous adjuvants, it will be of interest to determine if, like the hsp65 fusion proteins studied here, they can also activate dendritic cells directly and elicit CD8 T cell production in the absence of CD4 T cells.

### Hsp Fusion Protein Stimulation of CD8 T Cell Production Does Not Depend upon CD4 T Cells

Prior to the present study, one way to account for the ability of hsp fusion proteins to stimulate CD8 T cell production was to invoke a key role for CD4 T cells. Thus, a vigorous CD4 T cell response to peptides from the hsp moiety could activate dendritic cells and amplify an otherwise marginal CD8 T cell response to peptides from the fusion partner (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). This possibility is supported by older evidence that hsp65 can serve as an effective carrier molecule in the classic sense; i.e., when chemically coupled to nonimmunogenic hapten-like molecules (polysaccharides, a malarial peptide), the conjugates elicited IgG antibodies to the adducts in responses that were presumably T cell dependent (Barrios et al., 1992). This mechanism is clearly not essential, because $CD4^{-/-}$ mice injected with hsp65-P1 produced cytolytic CD8 T cells to the fusion partner's peptide. Nevertheless, it is entirely possible that in normal animals the response may be enhanced by CD4 T cells specific for peptides derived from the hsp moiety.

Previous efforts to determine whether CD4 T cells are essential for CD8 T cell responses to various immunogens and immunization strategies have yielded diverse results. With some epitopes, e.g., minor histocompatibility antigens, CD8 T cell responses could not be elicited in $CD4^{-/-}$ mice (Di Rosa and Matzinger, 1996), but with more potent immunogens (e.g., lymphocytic choriomeningitis virus or a murine herpes virus) or high doses of particulate antigens, CD8 CTL responses in $CD4^{-/-}$ mice were virtually the same as those in normal mice (Rahemtulla et al., 1991; Rock and Clark, 1996; Stevenson et al., 1998). That CD4 T cells are not required for the CD8 T cell response to hsp65-P1 raises the possibility that hsp fusion proteins are relatively potent immunogens for CD8 T cells.

### The Hsp Moiety in Hsp Fusion Proteins

In the several hsp fusion proteins examined here, the only common element is hsp65. The question arises as to how the hsp moiety can directly activate dendritic cells (and thereby elicit CD8 CTL production) regardless of wide variations in length and sequence of the fusion partners. It is particularly notable, in contrast, that unmodified (native) hsp65 lacks this critical activity. It may be that in the fusion proteins the hsp moiety adopts a particular conformation or displays a linear sequence, peptide motif, or pattern that is (1) necessary for eliciting the dendritic cell response, (2) retained despite wide variations in the fusion partner sequences, and (3) absent or masked in unmodified (native) hsp65. A detailed structural comparison between the various fusion proteins and native hsp65 will be required to distinguish between these possibilities.

The intensity of current interest in CD8 vaccines for HIV-1 and other persistent intracellular pathogens as well as for cancer cells is reflected in recent studies of diverse genetic vaccines and of several bacterial toxins fused to antigenic peptides or polypeptides as stimulators of CD8 CTL production. For example, nonapeptide sequences inserted into a truncated subunit of anthrax

toxin or pertussis toxin could elicit CD8 CTL in vivo (Ballard et al., 1996) and render target cells susceptible to lysis by cognate CD8 CTL in vitro (Goletz et al., 1997; Carbonetti et al., 1999). These and other bacterial toxins have evidently acquired through evolution the capacity to cross mammalian cell membranes and gain access to the cell cytosol, where they exert their lethal effects. While the judicious linkage of small peptides allows these toxin subunits to retain their ability to traverse membranes, the need to preserve this special property may limit the size and sequence diversity of the fusion elements that can be accommodated. For the hsp fusion proteins, in contrast, there appear so far to be no constraints to their effectiveness as CD8 immunogens by the length or sequence of the fusion partners. Because large fusion partners, e.g., the equivalent of a typical protein domain, are likely to encompass many potential epitopes for diverse MHC class I molecules, the hsp fusion proteins as a class warrant further study as candidate vaccines for use with populations of MHC-disparate individuals.

## Experimental Procedures

### Mice, CTL Clones, and Cell Lines

C57BL/6 (H-2$^b$), CD4-deficient (CD4 tm1Mak, H-2$^b$), and C3H/HeJ mice (H-2$^k$) were obtained from the Jackson Laboratories, maintained in barrier cages under specific pathogen-free conditions, and immunized between 4 and 10 weeks of age. 2C TCR transgenic mice (H-2$^b$) contain the rearranged transgenes encoding the $\alpha\beta$TCR from a 2C CTL clone (Sha et al., 1988). 2C TCR transgenic mice deficient for the recombination activating gene-1 (termed 2C/RAG) (Manning et al., 1997) were used as a source of naive T cells for in vitro assays (Cho et al., 1999). The 2C CTL clone L3.100 has been previously described (Sykulev et al., 1998). EL4 cells were obtained from the American Type Culture Collection and T2-K$^b$ cells were a generous gift from Peter Creswell, Yale University. PBS is (in mM) 140 NaCl, 2.7 KCl, 10 Na, $KPO_4$, PH 7.2.

### Plasmids, Peptides, and Proteins

In the P1 polypeptide, the sequences flanking the N and C termini of the SYRGL octapeptide (Figure 1A), from ovalbumin (ova251–257) and $\alpha$-ketoglutaraldehyde dehydrogenase, respectively, were modified by addition of a lysine residue penultimate to the N terminus (out of ubiquitination considerations) (Eisenlohr et al., 1992; York and Rock, 1996), and an isoleucine and a tyrosine residue were added at the N and C termini for cloning purposes. Complementary oligonucleotides encoding P1 were synthesized and cloned into a mammalian expression vector, VR1055 (Vical), and subsequently subcloned as an in-frame fusion at the 3′ end of the *M. bovis* BCG hsp65 gene (hsp65-P1) into the bacterial expression vector pET28A$^+$ (Novagen). The P1 sequence was also subcloned into the 3′ end of the gene encoding *E. coli* maltose binding protein in pMAL-p2E, using the pMAL protein fusion system (New England Biolabs) as well as into the mammalian expression vector pCIneo (Promega). All hsp65 fusion proteins used in this study as well as the unmodified hsp65 were produced as recombinant proteins in *E. coli*. They were purified under denaturing conditions from the soluble fraction of bacterial lysates and fractionated successively on butyl-Sepharose, Q-Sepharose, Ni-Sepharose when applicable, and finally by dialysis against PBS. Mal-P1 was purified by amylose affinity chromatography (New England Biolabs).

SDS-PAGE analysis of purified hsp65-P1 revealed a major species at 67.5 kDa, which was shown to be hsp65-P1 by Western analysis, using anti-mycobacterial hsp65–specific antibody (StressGen), and by electrospray mass spectrometry (MIT Biopolymer Laboratory). Mal-P1 was also subjected to amino acid analysis and SDS-PAGE to confirm molecular weight (45.4 kDa) and purity. P1 and SYRGL peptides were synthesized by the MIT Biopolymers Laboratory. Protein concentrations were estimated by bicinchoninic acid or amino acid analyses and were expressed in molar terms to facilitate comparisons between proteins and polypeptides of differing molecular masses. Endotoxin concentrations of recombinant protein preparations were determined by the Limulus assay, using reagents and conditions according to Associates of Cape Cod. Peptide concentrations were estimated by weight per volume or based on amino acid analyses.

### Antibodies and Flow Cytometry

Flow cytometry was carried out on a FACScaliber, using CellQuest software (Becton Dickinson). Unlabeled or FITC-, PE-, allophycocyanin-, or biotin-labeled antibodies against CD69, CD4, CD8, CD11c, CD11b, GR.1, B7.2, B220, or MHC class I (H-2$^b$) as well as secondary antibodies and streptavidin labeled with allophycocyanin or PE were obtained from PharMingen. 1B2, a clonotypic antibody that recognizes 2C TCR (Kranz et al., 1984), was purified from the 1B2 hybridoma and biotinylated using biotinamidocaproate N-hydroxysuccinimide ester (Sigma). The antibody, Y3, is cross-reactive with MHC class I from H-2$^b$ (K$^b$) and H-2$^k$ haplotypes. It was affinity purified from culture supernatants from the Y3 hybridoma (obtained from American Type Culture Collection) and labeled with fluorescein using fluorescein isothiocyanate.

### Generation of Bone Marrow–Derived Dendritic Cells and Isolation of Antigen Presenting Cells and Naive 2C T cells

To generate bone marrow–derived dendritic cells from C57BL/6 (or C3H) mice, bone marrow was flushed from the femur and tibia, red blood cells were lysed, and the remaining cells were cultured at $10^6$ cells/ml in RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 50 $\mu$m $\beta$-mercaptoethanol, 100 U/ml penicillin, and 100 $\mu$g/$\mu$l streptomycin) containing 20 ng/ml murine GM-CSF (R&D Systems). The medium was replaced on days 2 and 4, and on day 6 the cells (immature dendritic cells) were harvested for use.

In vitro assays were performed with purified cell populations unless otherwise noted. Magnetic cell sorting (MACS) was carried out according to the manufacturer's instructions (Miltenyi Biotec). Dendritic cells (splenic or bone marrow derived) were isolated by positive sorting using anti-CD11c antibody (purity ranged from 70%–97%). Peritoneal lavage macrophages were purified by treating them with biotinylated antibodies specific for CD11c, GR.1, and B220, followed by washing and incubating them with magnetic microbeads coated with anti-CD4 or anti-CD8 antibodies or with streptavidin and then passing them over a negative sorting column. Macrophage purity was typically >90%. For purification of 2C T cells from 2C/RAG transgenic mice, lymph node and spleen cells were coated with anti-CD8 magnetic beads (an average of two beads per cell) and positively sorted as above (purity >93%). The purification procedures did not activate APC or T cells as shown by flow cytometry: APC showed no increase in B7.2, MHC class I, or cell diameter, and T cells showed no CD69 upregulation after 24 hr in culture. There was also no significant [$^3$H]thymidine incorporation by T cells after 48 hr incubation.

### Cytolytic T Cell Assays

Unless otherwise noted, $^{51}$Cr-labeled T2-K$^b$ cells were used as target cells. They were incubated with effector cells derived from either fusion protein–injected mice or from cultured 2C T cell clones for 4 hr in the presence or absence of SYRGL (1 $\mu$M). Specific lysis was calculated as follows: [(experimental counts − spontaneous counts)/(total counts − spontaneous counts)] × 100.

To assess the ability of various APC to process hsp65-P1, dendritic cells and macrophages were used as target cells. Each of these cell populations was purified by MACS and then $^{51}$Cr labeled for 1 hr at 37°C. The labeled cells were then incubated with hsp65-P1 together with the 2C CTL clone (L3.100) at a CTL:target cell (E:T) ratio of 5:1. Assays were performed in triplicate using 96-well round-bottom plates, and cell supernatants were counted in a $\gamma$ spectrometer after 4 hr. Specific lysis was calculated as above.

### Transient Transfection and Antigen Processing Assays

EL4 cells ($5 \times 10^6$) were electroporated with 15 $\mu$g of the parent plasmids or plasmids containing the genes for P1 (in VR1055) or

hsp65-P1 (in pCIneo). Forty-eight hours after transfection, the cells were subjected to centrifugation in Ficoll-Paque (Pharmacia Biotech) (2200 rpm, 20 min), and $10^6$ live cells were incubated with an equal number of splenocytes from naive 2C/RAG mice. After 18 hr the cells were stained with 1B2, anti-CD69, and anti-CD8 antibodies (labeled with FITC, PE, and allophycocyanin, respectively), and 2C T cells were evaluated for upregulation of CD69 by flow cytometry, gating on propidium iodide–negative, $1B2^+CD8^+$ cells.

**Naive 2C T Cell Responses to Dendritic Cells and Macrophages and Dendritic Cell Activation Assays**

Purified dendritic cells and macrophages were incubated with various concentrations of proteins or peptides in 96-well ($5 \times 10^4$ cells/well) or 48-well ($1.5 \times 10^5$ cells/well) flat-bottom plates for 24 hr at 37°C. The following day an equal number of purified naive 2C T cells were added to each well (final volume = 200 μl for the 96-well plates, 600 μl for the 48-well plates). After 18 hr, the 48-well plates were separated into (1) cell pellets to analyze 2C T cells for expression of the acute activation marker CD69 by flow cytometry, gating on propidium iodide–negative, $1B2^+CD8^+$ cells and (2) cell supernatants to measure IL-2 secretion (in triplicate, using HT2 cells in a standard bioassay) (Watson, 1979). After 48 hr, the 96-well plates were assayed for IFNγ secretion (using 50 μl of cell supernatants and a capture ELISA assay [R&D Systems]) and for T cell proliferation (1 mCi [$^3$H]thymidine [NEN] was added per well, and 16 hr later the cells were harvested to measure [$^3$H]thymidine incorporation). Where indicated, 1B2 Fab fragments were added to naive 2C T cells at a final concentration of 25 μg/ml. Immature bone marrow–derived dendritic cells (day 6 of culture) were purified by magnetic sorting (>95% $CD11b^+CD11c^+$) and incubated ($2.5 \times 10^5$ cells/well in 96-well round-bottomed plates) with various fusion proteins or control proteins. The following day, cells were analyzed by flow cytometry for expression of B7.2 and MHC class I and II molecules, gating on propidium iodide–negative, $CD11c^+$ cells.

**Acknowledgments**

We are grateful to Carol McKinley for skillful technical help; Nir Hacohen for advice about dendritic cells; Glenn Paradis for help with flow cytometry; Richard Cook of the MIT biopolymer laboratory for peptide synthesis, amino acid analyses, and mass spectrometry; Evelyn Wiebe for endotoxin assays; and Jun Liu and Satoshi Sugawa for helpful discussions. We are grateful to Vical for the VR1055 plasmid. B. K. C. was supported by a National Institutes of Health training grant (5T32-AI-07463) and D. P. by a Stressgen postdoctoral fellowship. This work was supported in part by a National Institutes of Health Cancer Center core grant (CA-14051) and National Institutes of Health research grants (CA-60686, AI-44477, and AI-44478).

Received September 27, 1999; revised January 27, 2000.

**References**

Anthony, L., Wu, H., Sweet, H., Turnnir, C., Boux, L., and Mizzen, L. (1999). Priming of $CD8^+$ CTL effector cells in mice by immunization with a stress protein-influenza virus nucleoprotein fusion molecule. Vaccine *17*, 373–383.

Ballard, J., Collier, R., and Starnbach, M. (1996). Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope in vivo. Proc. Natl. Acad. Sci. USA *93*, 12531–12534.

Barrios, C., Lussow, A., Van Embden, J., Van der Zee, R., Rappuoli, R., Costantino, P., Louis, J., Lambert, P., and Del Giudice, G. (1992). Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guerin priming. Eur. J. Immunol. *22*, 1365–1372.

Bennett, S., Carbone, F., Karamalis, F., Flavell, R., Miller, J., and Heath, W. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signaling. Nature *393*, 478–480.

Carbonetti, N., Irish, T., Chen, C., O'Connell, C., Hadley, G., McNamara, U., Tuskan, R., and Lewis, G. (1999). Intracellular delivery of a cytolytic T-lymphocyte epitope peptide by pertussis toxin to major histocompatibility complex class I without involvement of the cytosolic class I antigen processing pathway. Infect. Immun. *67*, 602–607.

Cho, B., Wang, C., Sugawa, S., Eisen, H., and Chen, J. (1999). Functional differences between memory and naive CD8 T cells. Proc. Natl. Acad. Sci. USA *96*, 2976–2981.

Demangel, C., Bean, A., Martin, E., Feng, C., Kamath, A., and Britton, W. (1999). Protection against aerosol Mycobacterium tuberculosis infection using Mycobacterium bovis Bacillus Calmette Guerin-infected dendritic cells. Eur. J. Immunol. *29*, 1972–1979.

Di Rosa, F., and Matzinger, P. (1996). Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. J. Exp. Med. *183*, 2153–2163.

Eisenlohr, L., Yewdell, J., and Bennink, J. (1992). Flanking sequences influence the presentation of an endogenously synthesized peptide to cytotoxic T lymphocytes. J. Exp. Med. *175*, 481–487.

Falk, K., Rotzschke, O., and Rammensee, H. (1992). Specificity of antigen processing for MHC class I restricted presentation is conserved between mouse and man. Eur. J. Immunol. *22*, 1323–1326.

Goletz, T., Klimpel, K., Arora, N., Leppla, S., Keith, J., and Berzofsky, J. (1997). Targeting HIV proteins to the major histocompatibility complex class I processing pathway with a novel gp120-anthrax toxin fusion protein. Proc. Natl. Acad. Sci. USA *94*, 12059–12064.

Gromme, M., Uytdehaag, F., Janssen, H., Calafat, J., van Binnendijk, R., Kenter, M., Tulp, A., Verwoerd, D., and Neefjes, J. (1999). Recycling MHC class I molecules and endosomal peptide loading. Proc. Natl. Acad. Sci. USA *96*, 10326–10331.

Henderson, R., Watkins, S., and Flynn, J. (1997). Activation of human dendritic cells following infection with Mycobacterium tuberculosis. J. Immunol. *159*, 635–643.

Huang, Q., Richmond, J.F.L., Suzue, S., Eisen, H.N., and Young, R.A. (2000). In vivo cytotoxic T lymphocyte elicitation by mycobacterial heat shock protein 70 fusion proteins maps to a discrete domain and is CD4+ T cell independent. J. Exp. Med. *191*, 403–408.

Konecny, P., Stagg, A., Jebbari, H., English, N., Davidson, R., and Knight, S. (1999). Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro. Eur. J. Immunol. *29*, 1803–1811.

Kovacsovics-Bankowski, M., and Rock, K.L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science *267*, 243–245.

Kranz, D., Sherman, D., Sitkovsky, M., Pasternack, M., and Eisen, H. (1984). Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera. Proc. Natl. Acad. Sci. USA *81*, 573–577.

Manning, T., Rund, L., Gruber, M., Fallarino, F., Gajewski, T., and Kranz, D. (1997). Antigen recognition and allogeneic tumor rejection in $CD8^+$ TCR transgenic/RAG(−/−) mice. J. Immunol. *159*, 4665–4675.

Nair, S., Wearsch, P., Mitchell, D., Wassenberg, J., Gilboa, E., and Nicchitta, C. (1999). Calreticulin displays in vivo peptide-binding activity and can elicit CTL responses against bound peptides. J. Immunol. *162*, 6426–6432.

Poltorak, A., He, X., Smirnova, I., Liu, M., Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Rahemtulla, A., Fung-Leung, W., Schilham, M., Kundig, T., Sambhara, S., Narendran, A., Arabian, A., Wakeham, A., Paige, C., Zinkernagel, R., et al. (1991). Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. Nature *353*, 180–184.

Rescigno, M., Citterio, S., Thery, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., and Ricciardi-Castagnoli, P. (1998). Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. Proc. Natl. Acad. Sci. USA *95*, 5229–5234.

Ridge, J., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. Nature *393*, 474–478.

Rock, K. (1996). A new foreign policy: MHC class I molecules monitor the outside world. Immunol. Today *17*, 131–137.

Rock, K., and Clark, K. (1996). Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway. J. Immunol. *156*, 3721–3726.

Schoenberger, S., Toes, R., van der Voort, E., Offringa, R., and Melief, C. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480–483.

Sha, W., Nelson, C., Newberry, R., Kranz, D., Russell, J., and Loh, D. (1988). Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. Nature *335*, 271–274.

Sigal, L.J., Crotty, S., Andino, R., and Rock, K.L. (1999). Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. Nature *398*, 77–80.

Srivastava, P. (1993). Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune response to cancer and in antigen presentation. Adv. Cancer Res. *62*, 153–177.

Stevenson, P., Belz, G., Altman, J., and Doherty, P. (1998). Virus-specific CD8(+) T cell numbers are maintained during gamma-herpesvirus reactivation in CD4-deficient mice. Proc. Natl. Acad. Sci. USA *95*, 15565–15570.

Suto, R., and Srivastava, P.K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperone peptides. Science *269*, 1585–1588.

Suzue, K., Zhou, X., Eisen, H.N., and Young, R.A. (1997). Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. Proc. Natl. Acad. Sci. USA *94*, 13146–13151.

Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J., and Eisen, H.N. (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity *4*, 565–571.

Sykulev, Y., Vugmeyster, Y., Brunmark, A., Ploegh, H., and Eisen, H. (1998). Peptide antagonism and T cell receptor interactions with peptide-MHC complexes. Immunity *9*, 475–483.

Udaka, K., Tsomides, T., and Eisen, H. (1992). A naturally occurring peptide recognized by alloreactive $CD8^+$ cytotoxic T lymphocytes in association with a class I MHC protein. Cell *69*, 989–998.

Udaka, K., Wiesmuller, K., Kienle, S., Jung, G., and Walden, P. (1996). Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. J. Immunol. *157*, 670–678.

Watson, J. (1979). Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. J. Exp. Med. *150*, 1510–1519.

York, I.A., and Rock, K.L. (1996). Antigen processing and presentation by the class I major histocompatibility complex. Annu. Rev. Immunol. *14*, 369–396.